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Molecular Characterisation and Plasmid Profiling of Hydrocarbon Utilizing Bacteria Isolates from Wetlands in Rivers State, Southern Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Wetlands can intercept runoff from surfaces prior to reaching open water and remove pollutants through physical, chemical, and biological processes thereby protecting and preserving the environment. Because of unsustainable oil exploration activities, most wetlands in Rivers State, Southern Nigeria have suffered severe petroleum-damaged ecosystems. This research was carried out to characterize and identify the hydrocarbon utilizing bacteria associated with crude oil polluted wetlands and to screen for the presence of plasmids that could confer resistance to antibiotics using both cultural and molecular methods. Soil samples were collected from three different wetlands across the state with hand auger at two depths of 0-15cm and 15-30cm twice monthly for three months. The presence of microbial activity was determined by the enumeration and isolation of total heterotrophic and hydrocarbon utilizing bacteria. Eight (8) most occuring hydrocarbon utilizing bacterial isolates were isolated and identified culturally and phenotypically from the 54 wetland soil samples. These bacteria isolates were confirmed to be *Bacillus flexus, Bacillus subtilis, Lysinibacillus macroides, Staphylococcus aureus, Chryseobacterium aquifrigidense, Pseudomonas aeruginosa* and *Salmonella enterica* molecularly via sequencing of the 16S rRNA gene. The most common bacteria isolated were *Bacillus species*, followed by *Pseudomonas* at a dilution of 10^6. Seven (7) out of the eight (8) isolates (except *Salmoella enterica*) showed the presence of the 25kb plasmids at various intensities.

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Keywords: Wetlands; microorganisms; crude oil pollution; plasmids; molecular characterization.

1. INTRODUCTION

Wetland is an ecosystem that arises when accumulated by water produces soils dominated by anaerobic processes, which in turn, forces the biota, particularly rooted plants, to adapt to flooding" (Keddy, 2010). Wetland ecosystems are among the most important in the world, providing a diverse range of ecosystem services vital to human well-being (Barbier *et al.,* 1997; RCS, 2007).

The Niger Delta area of Rivers state is one of the most important wetland ecosystem in the world. Because of unsuitable oil exploration activities, the Niger Delta has suffered severe petroleum-damaged ecosystems [1]. Due to the influence of the tides and floods from rains, spilt oil is rapidly distributed over large areas and remobilized with rising tides. The oil originates from leaking pipelines, well heads and flow stations and through transport of mostly stolen oil from illegal tapping of the wells and from artisanal refining under very primitive conditions (Linden et al., 2013) [2]. The spills have resulted in the contamination of wetlands, penetrated into soils down to several meters, water wells and has caused serious concern from a health perspective. The use of hydrocarbon utilizing microorganisms to remediate crude oil spills has proven to be a promising solution to such environmental problems [3]. Microorganisms are present in high amounts in wetland environments and these microbes are capable of a number of important functions ranging from hydrocarbon degradation, nitrogen fixation, denitrification, iron and sulphate reduction [4]. Petroleum hydrocarbons can be biodegraded by various groups of microorganisms, bacteria however are the most active group of hydrocarbon degraders, and they act as primary degraders of hydrocarbon in an environment [5]. Hydrocarbon utilizing bacteria are ubiquitouos in the environment and their use in bioremediation exploits their ability to utilize organic contaminants as an economical, efficient, versatile and environmentally friendly treatment.

Wetland soils in Rivers State, Southern Nigeria are polluted regularly by diverse petroleum products due to the local refining processes ("kpo fire") which are carried out along most water front in the state. Oil spillage is a major cause of loss of wetlands. Indiscriminate oil activities, including the exploration, exploitation and transportation of crude oils in water can also lead to spillages on wetland soils, making them unsuitable for agricultural cultivation. The threats have induced changes that eroded the ecological and socio-economic values as well as services derived from the wetland (Ramsar, 2011). Most of these activities are done out of ignorance, paucity of information, lack of awareness, understanding, advocacy on waste management and lack of understanding of wetland values. Very often, wetlands are drained, then houses and other buildings are built on the land that used to house diverse species of microorganisms, birds, fish and amphibians. The extent of the damage caused by oil spillage to the microbial population is dependent on several factors including: the composition of the microbial community prior to the contamination, chemical composition of the contaminant and the physicochemical factors of the contaminated sites.

Research has shown that bacteria such as *Pseudomonas, Alcaligenes, Bacillus, Corynebacterium, Klebsiella, Acinetobacter, Flavobacterium, Alcanivorax* and *Proteus species* have the ability to utilize hydrocarbon [6,7]. These microorganisms have been characterized both conventionally and molecularly by researchers such as Brito *et al*. [8], Babu *et al*. [9] and Subathra *et al*. [10].

Enumeration of hydrocarbon utilizing bacteria population in polluted environments using conventional methods often provides probationary phenotypic identities of the cultured isolates. Recent advances in microbial ecology has made it possible to combine molecular and conventional methods to describe microorganisms (bacteria) and their ability to utilize hydrocarbon in polluted environments [8]. For the past two decade microbiologists have relied solely on the use of 16S rRNA gene sequencing for identification, classification and estimation of bacterial diversity/dynamics in environmental samples through PCR and DNA sequencing [11]. PCR is an extremely sensitive technique that allows the amplification of millions of copies of a portion of a desired gene, entire gene or gene clusters with high precision within three (3) to four (4) hours with the help of a DNA polymerase enzyme and specific primers [12]. One of the major reasons for prolonged negative impact of oil spill on the environment could probably be due to absence of adequate and qualitative scientific baseline data which are

required to provide informed and quick response to emergent environmental challenges.

Plasmids are extra-chromosomal materials which are able to effect the production of β-lactamases. Some scholars have suggested that these β-lactamases have not only been active against β-lactam drugs but also on aminoglycosides and quinolones which are non-β-lactam drugs; this have more destroying effect on patients who are immunocompromised and makes treatment of illnesses difficult [13]. Plasmids are often implicated in increasing drug resistance as they are able to transfer the genes both within species and between different species [14,15]. Mechanisms of antibiotic resistance include structural modification of the target, degradation of the drug by enzymes and efflux of antibiotics [16]. Conversely, the genes responsible for resistance are either located on the chromosome or on the plasmid. This provides a medium for the quick spread of resistance genes than mutation and vertical evolution [17]. Plasmid profiling has proved to be relevant in the epidemiologic study of drug resistance as this explains the pattern, occurrence and likely future picture of the resistance when linked with some parameters [18,19]. It also aids in surveillance in relating strains with outbreaks and their spread [20]. The objective of this study was to characterize the culturable indigenous hydrocarbon utilizing bacteria using conventional culture-dependent and molecular approach as well as to assess the presence of plasmids in the bacterial isolates. This approach is expected to increase the possibilities of developing models and strategies for the bioremediation of hydrocarbon pollutants in the environment.

2. MATERIALS AND METHODS

2.1 Description of Study Area

This study area was carried out in three different wetlands in Rivers State, Southern Nigeria and the study stations were Iwofe ($4^0$48'46.551" N, $6^0$56'12.0906" E), Eagle Island ($4^0$47'47.302"N, $6^0$58'24.5496" E) and Chokocho ($4^0$59'53.75688" N, $7^0$3'39.93084" E).

2.2 Sample Collection

Wetland soil samples were collected under aseptic conditions with the aid of a hand auger at two depths (0-15cm and 15-30cm) and three positions; one meter apart in the three wetlands (Iwofe IF; Eagle Island EI and Chokocho CK) in order to obtain composite samples. Permission from the Local Government authority was not required to obtain the soil samples from the wetlands. A total of fifty-four soil samples were collected for a period of three months from the three wetland stations in Rivers State. The soil samples were put in sterile polyethylene bags and conveyed to the Microbiology Laboratory of the Department of Microbiology, Rivers State University, Port Harcourt for analyses within 24 hours.

2.3 Serial Dilution

The presence of various microorganisms in the soil samples were identified using standard microbiological procedures. One gram each of the soil samples was separately added to 9 ml of 0.1% peptone water diluents to give a 10^{-1}dilution w/v. Further serial 10- fold (v/v) dilutions were made by transferring 1ml of the original solution to freshly prepared peptone water diluents (9ml) up to a range of 10^{-5} dilutions. Similarly, ten grams of the soil sample was added to 90ml of sterile distilled water to get an aliquot.

2.3.1 Enumeration of total culturable heterotrophic and hydrocarbon utilizing bacteria

Total culturable heterotrophic bacteria (THB) were determined using spread plate method on nutrient agar (NA) while culturable hydrocarbon utilizing bacteria (HUB) were enumerated by vapour phase transfer method using mineral salt agar according to Hamamura *et al*. [21]. Identification of each hydrocarbon utilizing bacterial isolate was further conducted using biochemical tests such as Gram staining, Citrate Utilization, Catalase, Methyl Red, Indole, Voges Proskaeur and sugar fermentation tests [22,23]. Individual colonies were phenotypically identified using Bergey's Manual for Determinative Bacteriology [24].

2.4 Total Heterotrophic Bacteria (THB) Count

Aliquots (0.1 ml) of various dilutions were inoculated onto the surface of dried nutrient agar in triplicates and spreading with flamed bent glass spreader and incubated at 37□ C for 24 hours. Total Heterotrophic Bacteria from the soil samples was enumerated as described by Prescott *et al* (2005). Bacterial Colonies that appeared on the nutrient agar plates were counted and the mean expressed as cfu/g for the

soil samples [25]. The colony forming unit per gram sample was calculated using the formula below;

$$\text{CFU/ml} = \frac{\text{number of colonies}}{\text{Dilution x volume plated}}$$

2.5 Total Hydrocarbon Utilizing Bacteria (THUB) Count

The Vapour Phase Transfer method of Mills and Cowell (1978) was adopted to determine the population of hydrocarbon utilizing bacteria. Aliquots (0.1 ml) of the serially diluted samples were inoculated on Mineral Salt Agar media using the spread plate technique as described by Odokuma (2003). Sterile filter paper discs soaked in filter-sterilized crude oil which served as the only carbon source in the mineral salt agar was placed aseptically in the cover of the inoculated agar plates in duplicates. The plates were incubated for 5 days at room temperature (25^0C). After the incubation period, the number of colonies were counted and the mean of the colonies were determined in cfu/g.

2.5.1 Identification of bacterial isolates by conventional phenotypic method

The discrete bacteria isolated from the samples were characterized based on their cultural morphology which includes colour, texture, shape, size, elevation, etc. of the isolate while, biochemical characteristics which include test include; Gram' reaction, motility, catalyse, oxidase, spore formation, indole production, methyl red, citrate utilization, Voges Proskauer test and sugar fermentation of the discrete bacterial isolates were compared with the recommendation by Cowan and Steel (1994), for the identification of the bacterial isolates.

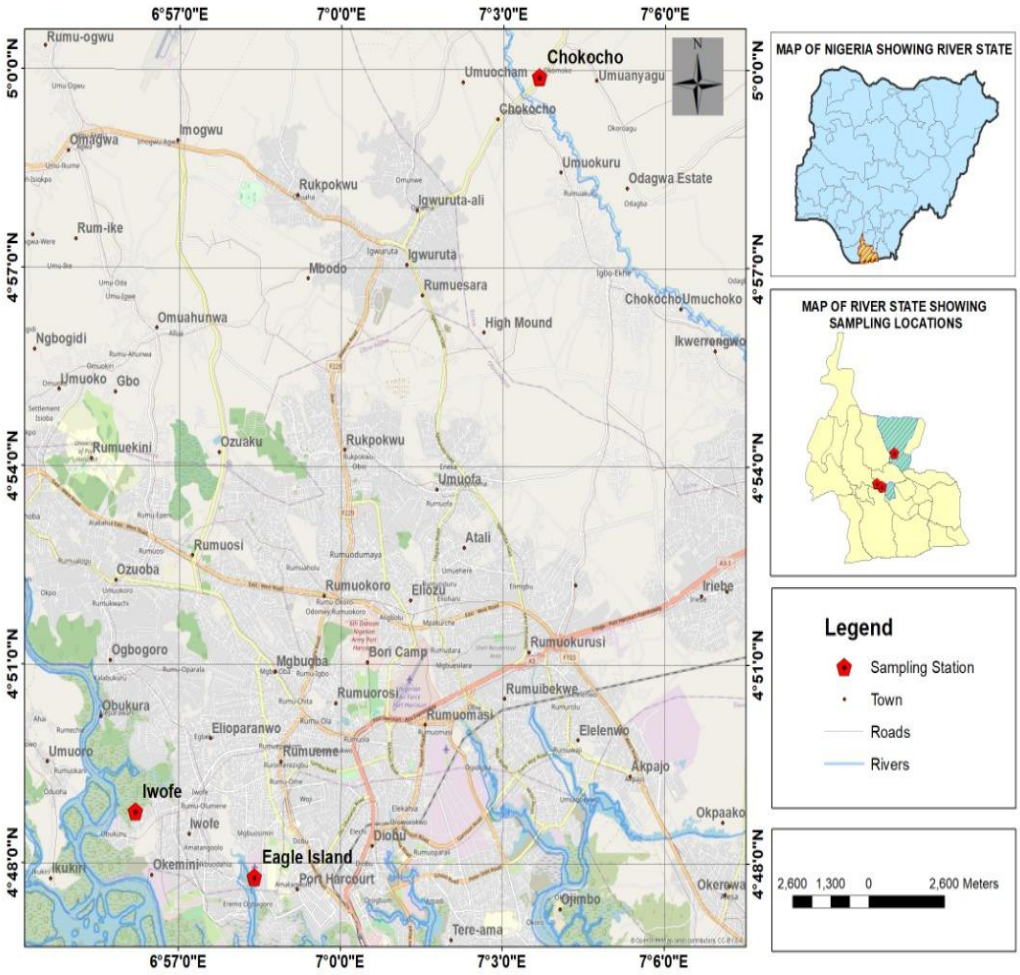

Fig 1. Map showing the three wetland sampling stations in this study

2.5.2 Purification of isolates for identification of bacterial isolates by conventional phenotypic methods

After incubation, pure isolates were obtained by picking (with sterile inoculation loop) distinct culturally and morphologically different colonies from the various nutrient agar plates. These were subjected to streaking on sterile nutrient agar in plates and subsequent sub-culturing until pure distinct colonies were formed for further identification (phenotypically and degradation screening).

2.5.3 Degradation screening: pure culture of the individual hydrocarbon utilizing isolates

Degradation screening was carried out to identify the microorganisms in the total heterotrophic population which possess the ability to degrade or utilize the hydrocarbon present in the wetland soils. Representative bacterial isolates were screened for oil degradation capability under aerobic conditions by inoculating a calibrated loopful of 18hour old culture of each bacterium into mineral salt agar using the vapour phase method in which crude oil served as the sole source of carbon [25]. A filter paper (Whattman's No. 1) saturated with crude oil was aseptically placed onto the covers of the petri dishes and inverted. The culture plates were incubated for 5 to 7 days at 37^0C. Isolated colonies were further purified by sub culturing onto on freshly prepared well dried nutrient agar plates and identified using biochemical test and microscopy [26,24]. Pure cultures of the individual hydrocarbon utilizing isolates were used for molecular characterization.

3. MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

3.1 DNA Extraction and Quantification

Boiling method was used for the extraction process as described by Bell *et al.* [27]. Pure culture of the individual hydrocarbon utilizing isolates from the THUB was put in Luria-Bertani (LB) Broth and incubated at 37^oC. 0.5ml of the broth culture of each bacterium in Luria Bertani (LB) was put into properly labeled Eppendorf tubes and filled to mark with normal saline, then centrifuged at 14000rpm for 3 minutes and the supernatant was decanted leaving the bacterial pellet at the base. This process was repeated 3 times. The cells were re-suspended in 500ul of normal saline and heated at 95^0C for 20 min. The heated bacterial suspension was cooled on ice (about 10minutes) and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml micro-centrifuge tube and stored at -20^oC for other down-stream reactions [27]. The extracted DNA's were quantified by using the Nanodrop 1000 Spectrophotometer as described by Olsen and Morrow (2012).

3.2 16S rRNA Gene Amplification

The 16srRNA Amplification was carried out using an ABI 9700 Applied Biosystems Thermal Cycler, method described by Srinivasan *et al*. (2015). The 16s rRNA region of the rRNA gene of the hydrocarbon utilizing bacterial isolates were amplified using universal primers, forward primer; 27F: 5'-AGAGTTTGATCTGGCTCAG-3' and Reverse primer; 1492R: 5'-CGGTTACCTTGTTACGACTT-3' on ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 micro-litres for 35 cycles. The PCR mix includes: (Taq polymerase, DNTPs, $MgCl_2$), the primers at a concentration of 0.5uM and the extracted DNA as template, Buffer 1X and water. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light trans-illuminator for a 1500bp amplicons (Srinivasan *et al*., 2015).

3.3 Gel Electrophoresis of the 16SrRNA

The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light trans-illuminator for a 1500bp amplicons (Srinivasan *et al*., 2015). The Agarose gel electrophoresis of the amplified 16SrRNA gene of eight (8) selected hydrocarbon utilizing bacterial isolates before sequencing, Lanes P1-P8 represent the 16SrRNA gene bands (1500bp) while lane M represents the 100bp molecular ladder (Fig. 2).

3.4 DNA Sequencing

Sequencing of the amplified product was carried out using the Big-Dye Terminator kit on a 3510 ABI sequencer. The sequencing was done at a final volume of 10ul, the components included 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5

x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows; 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4minutes (Srinivasan *et al*., 2015).

3.5 Phylogenetic Analysis

Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN prior to the edition of the obtained sequences using the bioinformatics algorithm Trace edit. MAFFT were used to align these sequences. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The evolutionary distances were computed using the Jukes-Cantor method [28].

4. RESULTS

The presence of microbial activity was determined by the enumeration and isolation of total heterotrophic and hydrocarbon utilizing bacteria carried out and presented in previous work of Chibuike *et al*. [29].

The Agarose gel electrophoresis of the amplified 16S rRNA gene of the hydrocarbon utilizing bacterial isolates before sequencing showed 16SrRNA gene bands (1500bp) (Fig. 2). The evolutionary distance between the bacterial isolates from this study and the accession numbers and their closest relatives on the phylogenetic tree is revealed on Fig. 3.

The agarose gel electrophoresis shows that seven out of the eight hydrocarbon utilizing bacterial isolates screened for plasmid had the presence of plasmid which may confer possible virulence to them (Fig 2).

5. DISCUSSION

This research is aimed at molecular characterization of hydrocarbon utilizing bacterial isolates which may have possible virulence due to the presence of plasmid. Molecular techniques have been employed recently, to facilitate the reliable identification of microorganisms. Amplification of sequences that is specific for an organism can be done through polymerase chain reaction. This research is aimed at molecular characterisation of hydrocarbon utilizing bacterial isolates which may have possible virulence due to the presence of plasmid.

Ranjard *et al*. (2000) reported that cultural techniques are no longer reliable in the identification of microorganisms due to anomalies and human errors, hence the use of molecular identification for microbial identification. Bacterial 16S rRNA sequences were aligned with BLAST algorithm of National Centre for Biotechnology Information (NCBI) database. Sequences aligned showed 100% similarity with those deposited in GenBank and the eight isolates were genotypically identified as *Bacillus flexus, Bacillus subtilis, Lysinibacillus macrolides, Staphylococcus aureus, Chryseobacterium aquifrigidense, Pseudomonas aeruginosa* and *Salmonella enterica*. The phenotypic characterization of the only four isolates was the same with the genotypic isolates identified (P1 to P4). P5 to P8 were identified genotypically to be different isolates from what was phenotypically identified by cultural methods. The use of 16S rRNA in the characterization of hydrocarbon utilizing microorganisms is more reliable and sensitive than culture-dependent techniques alone [8,5,9,30] and the results obtained in this investigation are consistent with past field studies [31,32].

The bacteria genera found are in line with a previous report by Eze *et al*. [33] who also isolated similar organisms. Some of the hydrocarbon utilizing bacteria isolated were either pathogenic with presence of plasmids (Fig 3) or enteric and could have found themselves in these wetlands as a result of poor sanitary conditions around the wetland environments. The bacteria could have adapted to this environment, thereby utilizing the hydrocarbons in the stream as a source of carbon resulting in their being hydrocarbonoclastic. All the eight bacterial isolates showed tolerance to hydrocarbon as they were able to grow in the mineral salt agar using the vapour vase method where crude oil was the only source of carbon.

Bacillus subtilis and *Psuedomonas aeruginosa* were the most dominant bacteria isolated across the three wetlands and they have been isolated from various ecological and geographical environments as hydrocarbon degraders [34-36].

Soil and marine hydrocarbon utilizing bacteria have been demonstrated to produce bioemulsifiers and biosurfactants which greatly enhance bioavailability by transporting hydrocarbons into cell through efficient uptake mechanisms [37] and as such species of *Pseudomonas* and *Bacillus* obtained in this study may have such potential [38,39]. The isolation of

high number of hydrocarbon utilizing microorganisms from petroleum-polluted environment is commonly taken as evidence that those organisms are the active hydrocarbon degraders in that environment. The microorganisms capable of surviving in such environment are those that have developed enzymatic and physiological responses that allow them use the hydrocarbons as substrates [40]. These findings have revealed that there is an appreciable population of active indigenous hydrocarbon utilizing bacteria in the three oil polluted wetlands which can be monitored and enhanced to bring about bioremediation in this area. The 16S rRNA gene marker employed in this study to identify and characterize bacteria isolates provided an efficient molecular approach to elucidate the bacterial population of the study area.

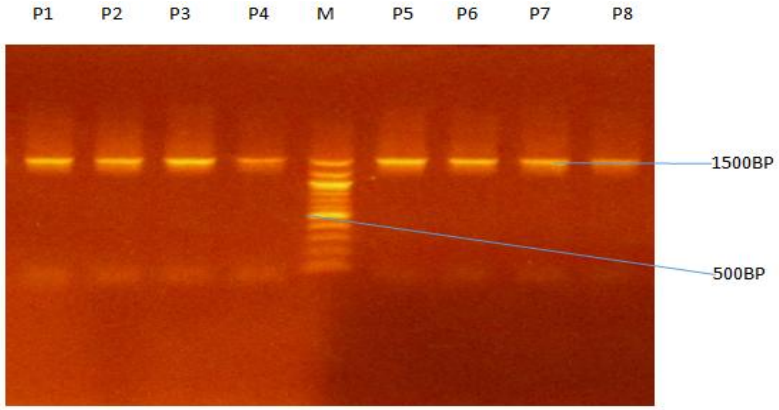

Fig. 2. Agarose Gel Electrophoresis showing the Amplified 16S rRNA Gene of Eight Hydrocarbon Utilizing Bacterial isolates; (Lanes P1-P8 represent the 16SrRNA gene bands (1500bp); Lane M represents the 100bp molecular ladder)

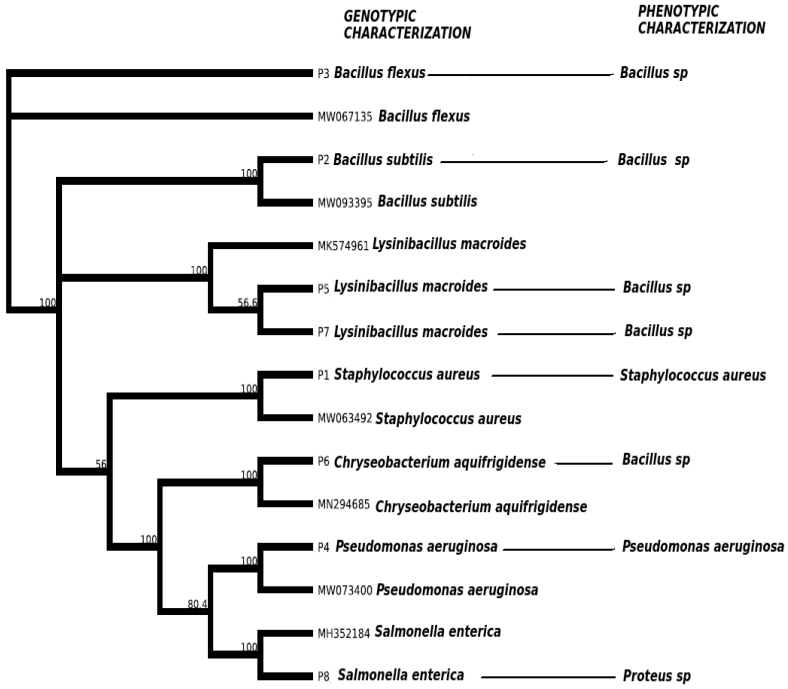

Fig. 3. Phylogenetic Tree showing the Evolutionary Distance between the Bacterial Isolates

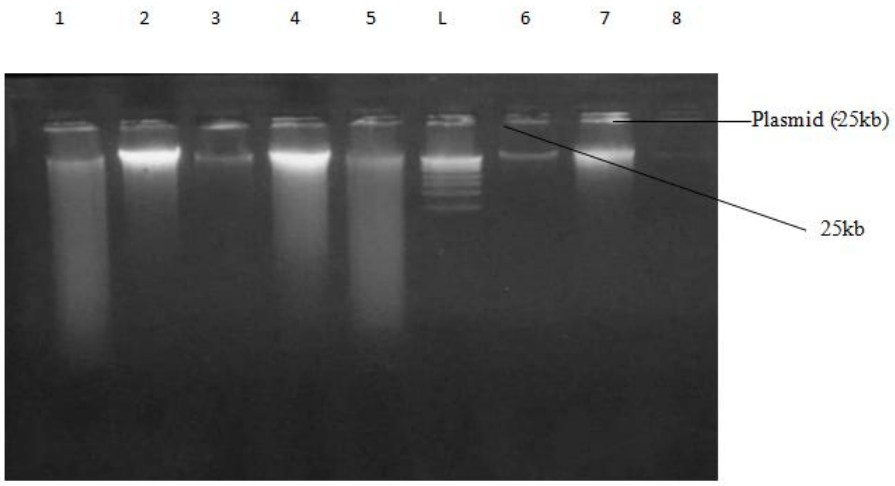

Fig. 4. Agarose Gel Electrophoresis Showing Single Plasmid Bands of Various Intensity from the Bacterial Isolates; (Lane L represents the 1kb molecular ladder; lane 1-8 represent plasmid of approximately 25kb in size)

6. CONCLUSION

From this study, it is evident that these three wetlands in Rivers State are indeed polluted. The high concentrations of these pollutants in the wetlands could have negative effects on animal and human health. The public should be enlightened on the health and environmental impact of incessant disposal of used petroleum products into the stream which may run off or seep into the surrounding wetland soils. This study also showed that these bacterial species isolated from the wetlands have great potentials for bioremediation of the hydrocarbons found in these environments.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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